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<b>(21) International Application Number:</b> PCT/US93/04581 <b>(22) International Filing Date:</b> 13 May 1993 (13.05.93)  <b>(30) Priority data:</b> 884,423 15 May 1992 (15.05.92) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 884,423 (CON) Filed on 15 May 1992 (15.05.92)  <b>(71) Applicant (for all designated States except US):</b> NORTH CAROLINA STATE UNIVERSITY [US/US]; Campus Box 7003, 103 Holladay Hall, Raleigh, NC 27695-7003 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> PETITTE, James, N. [US/US]; 620 Shadywood Lane, Raleigh, NC 27603 (US). YANG, Zengming [CN/US]; 17 Enterprise Street, Raleigh, NC 27607 (US).  <b>(74) Agents:</b> SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, Post Office Drawer 34009, Charlotte, NC 28234 (US).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> AVIAN EMBRYONIC STEM CELLS  <b>(57) Abstract</b>  A method of producing undifferentiated avian cells expressing an embryonic stem cell phenotype is disclosed. The method comprises collecting avian cells from an avian blastoderm prior to formation of the primitive streak, then depositing the avian cells in contact with a mouse fibroblast feeder cell layer, and then growing the avian cells on the mouse fibroblast feeder cell layer in the presence of a media containing leukemia inhibitory factor in a differentiation-inhibiting amount for a time sufficient to produce a sustained avian cell culture. Cell cultures produced by the aforesaid process and veterinary pharmaceutical formulations containing such cells are also disclosed.		

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## AVIAN EMBRYONIC STEM CELLS

Field of the Invention

The present invention relates to stem cells in general, and particularly relates to avian embryonic stem cells.

5

Background of the Invention

Embryonic stem cells (ESCs) were first cultured from mouse embryos using a feeder layer of mouse fibroblasts or media conditioned with buffalo rat liver cells. The established ESC lines from mouse embryos have a characteristic phenotype consisting of a large nucleus, a prominent nucleolus, and relatively little cytoplasm. Such cells can be grown relatively indefinitely using the appropriate culture conditions. They can be induced to differentiate in vitro using retinoic acid or spontaneously by removal of the feeder layer or conditioned media. In addition, these cells can be injected into a mouse blastocyst to form a somatic and germ line chimera. This latter property has allowed mouse ESCs to be used for the production of transgenic mice with specific changes to the genome. See M. Evans et al., *Nature* 292, 154 (1981); G. Martin, *Proc. Natl. Acad. Sci. USA* 78, 7638 (1981); A. Smith et al., *Developmental Biology* 121, 1 (1987); T. Doetschman et

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al., *Developmental Biology* 127, 224 (1988); A. Handyside et al., *Roux's Arch Dev. Biol.* 198, 48 (1989).

5 The active compound that allows the culture of murine embryonic stem cells has been identified as differentiation inhibiting activity (DIA), also known as leukemia inhibitory factor (LIF). See A. Smith, *J. Tiss. Cult. Meth.* 13, 89 (1991); J. Nichols et al., *Development* 110, 1341 (1990). Recombinant forms of LIF can be used to obtain ESCs from mouse embryos. See S. Pease et al.,  
10 *Developmental Biology* 141, 344 (1990).

Subsequent to the work with mouse embryos, several groups have attempted to develop stem cell lines from sheep, pig and cow. A few reports indicate that a cell line with a stem cell-like appearance has been  
15 cultured from porcine embryos using culture conditions similar to that used for the mouse. See M. Evans et al., PCT Application WO90/03432; E. Notarianni et al., *J. Reprod. Fert., Suppl.* 41, 51 (1990); J. Piedrahita et al., *Theriogenology* 34, 879 (1990); E. Notarianni et al.,  
20 *Proceedings of the 4th World Congress on Genetics Applied to Livestock Production*, 58 (Edinburgh, July 1990).

Few or no attempts have been made to date regarding the culture of embryonic stem cells from avian embryos. The main reason for this is that it is very  
25 difficult to establish a continuous line of chicken cells without viral or chemical transformation, and most primary chicken lines do not survive beyond 2-3 months. The culture of cells from the unincubated embryo has been more difficult, and under reported conditions such cells  
30 do not survive beyond two weeks. See E. Mitrani et al., *Differentiation* 21, 56-61 (1982); E. Sanders et al., *Cell Tissue Res.* 220, 539 (1981).

#### Summary of the Invention

35 We have developed a process that allows the culture of cells with an embryonic stem cell phenotype from the avian embryo. The development of this process

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was problematic. First, we attempted to culture chicken embryo cells on a chicken fibroblast feeder layer. This was not successful. Next, we attempted to culture chicken embryo cells on a mouse feeder layer. This was not successful either. We then attempted to culture chicken embryo cells with BRL conditioned media. This also was not successful. Finally, we cultured chicken embryo cells on a mouse feeder layer in the presence of conditioned media and obtained the cultured stem cells described herein.

A first aspect of the present invention is, accordingly, a method of producing undifferentiated avian cells expressing an embryonic stem cell phenotype. The method comprises collecting avian cells from an avian blastoderm prior to formation of the primitive streak, then depositing the avian cells in contact with a mouse fibroblast feeder cell layer, and then growing the avian cells on the mouse fibroblast feeder cell layer in the presence of a media containing leukemia inhibitory factor in a differentiation-inhibiting amount for a time sufficient to produce a sustained avian cell culture. The sustained avian cell culture consists essentially of undifferentiated avian cells having a large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype"). Typically, the undifferentiated avian cells are capable of maintaining the stem cell phenotype when grown on the mouse fibroblast feeder layer in the presence of the aforesaid media for at least three days, and even for 1, 2, 3, or 4 or more weeks.

A second aspect of the present invention is a sustained avian cell culture consisting essentially of undifferentiated avian cells having a large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype"). The cells are produced from ancestor cells isolated from an avian blastoderm prior to formation of the primitive streak.

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A third aspect of the present invention is a veterinary pharmaceutical formulation comprising sustained avian cells as described above with respect to sustained avian cultures in a pharmaceutically acceptable carrier.

While applicants do not wish to be bound to any particular theory of operation of the invention, it appears that the use of an avian feeder cell layer, which would appear the logical choice if one wished to obtain avian embryonic stem cells, actually encourages the cells being cultured to differentiate towards fibroblasts. The solution to this problem appears to be the use of the mouse feeder cell layer. This, however, appears to raise a second problem in that the mouse feeder cell layer alone is insufficient to produce a sustained culture. This second problem appears obviated by the use of a media containing leukemia inhibitory factor in combination with the mouse feeder cell layer.

The foregoing and other objects and aspects of the present invention are explained in greater detail in the figures herein and the specification set forth below.

#### Brief Description of the Drawings

Figure 1 shows chicken embryonic stem cells (passage 8) cultured on STO feeder cells in BRL-conditioned media 48 hours after passage. Several nests of cells with an embryonic stem cell-like phenotype can be seen.

Figure 2 shows a confluent layer of chicken embryonic stem cells ready for passage. Stem cell colonies have expanded to join each other.

#### Detailed Description of the Invention

The term "avian" as used herein refers to any avian species, including but not limited to chicken, turkey, duck, goose, quail and pheasant. Chicken is currently preferred.

The term "sustained" as used herein with respect to cells and cell cultures refers to a cell or cell culture capable of undergoing further cell division, even if the cells are eventually subject to senescence.

5 Avian embryos from which cells are obtained for carrying out the present invention are preferably in a stage prior to formation of the primitive streak, and most preferably in stage IX to XIV of development (i.e., a blastoderm). Such embryos may conveniently be obtained  
10 from eggs immediately after they are laid (referred to as "unincubated eggs"). Embryo cells are preferably collected from the central disk of the area pellucida. Other regions could be used, but this dilutes out the desired cells and requires subsequent isolation of  
15 desired cell colonies during growth in culture.

The feeder cell layer as used herein is constructed in accordance with procedures known in the art. As noted above, the feeder cell layer preferably consists of mouse fibroblast cells. STO fibroblasts are  
20 preferred, but primary fibroblasts are also suitable. Also, while the present invention has been described with respect to the use of mouse cell feeder layers, it is contemplated that feeder layers comprised of cells from other murine species (e.g., rat) or other mammalian  
25 species (e.g., ungulate, bovine, and porcine species) may also be used.

The media used in carrying out the present invention may be any suitable media containing leukemia inhibitory factor (LIF) in a differentiation-inhibiting  
30 amount. The media may be a conditioned media or a synthetic media containing recombinant LIF, both of which are known in the art. Conditioned media, and particularly BRL conditioned media, is currently preferred.

35 Cells from the unincubated avian embryo are seeded onto the feeder layer with conditioned media, and the avian cells give rise to nests of cells exhibiting a

stem cell phenotype. Unlike the case with mammalian stem cells, it currently appears necessary to have both a suitable feeder layer and conditioned media, since avian ESC-like cells do not appear to survive when transferred to a feeder-layer alone or to BRL-conditioned media alone. The avian embryo cells of the present invention can be cultured for at least one or two months, which is significantly greater than the usual two week life of primary cultures of cells from the unincubated avian embryo.

Cell cultures of the present invention may be formulated for administration to animals by dissociating the cells (e.g., by mechanical dissociation) and intimately admixing the cells with a pharmaceutically acceptable carrier (e.g., phosphate buffered saline solution). Avian cells in such formulations may be prepared to carry a heterologous DNA sequence into an avian subject in the manner described in greater detail below.

Stem cells of the present invention are useful, among other things, as a tool for the study of embryological development (i.e., by labelling the cells with a marker gene and observing their distribution after injection *in vivo*) and the production of transgenic poultry. They are useful in allowing the application homologous recombination to the production of transgenic poultry.

In avian species, certain donor cell types have been isolated that retain viability when injected into recipient embryos. See Etches et al., in Avian Incubation, Chapter 22, Butterworth Publishers (1990); Verrinder Gebbins et al., Fourth World Congress on Genetics Applied to Livestock Production, Edinburgh, (1990); Petitte et al., Development 108, 185-189 (1990)). These studies showed that blastodermal cells derived from Stage X embryos (embryo at oviposition) remained viable when transferred to comparable recipient Stage X embryos.



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The present invention provides a new method of altering the phenotype of a bird and the birds so produced with the avian embryonic stem cells disclosed herein. The method comprises transfecting avian embryonic stem cells as disclosed herein with the DNA sequence in vitro (e.g., by electroporation or transformation with a retroviral vector), and then injecting the transfected embryonic stem cells into an egg containing an embryonic bird (e.g., into the yolk sac or onto the chorioallantoic membrane, preferably into the subgerminal cavity, and preferably during early embryonic development (e.g., prior to day 2 or 3 of incubation, and most preferably prior to day 1 of incubation)), with the DNA sequence being effective to cause a change in phenotype in the bird after hatch (e.g., a change in growth rate, feed efficiency, disease resistance, or a combination of all of these factors). Preferably, the egg into which the DNA is introduced is incubated to hatch, and the bird so produced raised to at least an age at which the change in phenotype is expressed. It is of no deleterious consequence if the transformed embryo and bird is chimeric, so long as a physiological response is achieved in the animal after hatch sufficient to evoke the phenotypic change sought.

The mechanism of *in ovo* injection is not critical, but it is preferred that the method not unduly damage the tissues and organs of the embryo or the extraembryonic membranes surrounding it so that the treatment will not decrease hatch rate. A hypodermic syringe fitted with a needle of about 18 to 26 gauge is suitable for the purpose. Depending on the precise stage of development and position of the embryo, a one-inch needle will terminate either in the fluid above the chick or in the chick itself. A pilot hole may be punched or drilled through the shell prior to insertion of the needle to prevent damaging or dulling of the needle. If desired, the egg can be sealed with a substantially

bacteria-impermeable sealing material such as wax or the like to prevent subsequent entry of undesirable bacteria. It is envisioned that a high speed automated injection system for avian embryos will be particularly suitable for practicing the present invention. Numerous such devices are available, exemplary being the EMBREX INOVOJECT™ system (described in U.S. Patent No. 4,681,063 and 4,903,625 to Hebrank), and U.S. Patents Nos. 4,040,388, 4,469,047, and 4,593,646 to Miller. The disclosure of these references and all references cited herein are to be incorporated herein by reference. All such devices, as adapted for practicing the present invention, comprise an injector containing the embryonic stem cell as described herein, with the injector positioned to inject an egg carried by the apparatus with the DNA. In addition, a sealing apparatus operatively associated with the injection apparatus may be provided for sealing the hole in the egg after injection thereof.

The DNA sequence introduced in ovo with embryonic stem cells of the invention is, in general, a construct comprised of a promoter functional in avian cells and a gene encoding a peptide or protein operably linked to the promoter. Preferably, the protein or peptide is physiologically active and capable of producing a phenotypic change in the bird. In general, the DNA construct may be a linear DNA sequence (introduced into the embryonic stem cells of the invention by electroporation) or a sequence carried by a vector or other suitable carrier for transforming the embryonic stem cells of the invention, such as liposomes, calcium phosphate, or DMSO. Vectors, as discussed below, may be plasmids, viruses (including retroviruses), and phage, whether in native form or derivatives thereof.

Illustrative of genes encoding a protein or peptide are those which encode a protein or peptide selected from the group consisting of growth hormone, thyroid releasing hormone (TRH), Marek's MDX, and

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immunogenic recombinant antigens such as that for coccidiosis.

The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. See e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col 9 line 65; U.S. Patent No. 4,877,729 to Clark et al. at Col. 4 line 38 to Col 7 line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3 line 26 to Col. 14 line 12. Protocols for restriction endonuclease digestion, preparation of vectors, DNA purification and other such procedures were essentially as described in standard cloning manuals. See Sambrook et al., Molecular Cloning, a Laboratory Manual, (2d Ed., Cold Spring Harbor Press, New York (1989)).

A vector is a replicable DNA construct used herein to either amplify and/or express DNA encoding the gene of interest. A suitable expression vector will have controlling elements capable of expressing the cloned cDNA or genomic DNA placed in the correct orientation when the vector is introduced into the correct host. Such elements typically include but are not limited to a promoter region which interacts specifically with cellular proteins involved in transcription, enhancer elements which can stimulate transcription many-fold from linked heterologous promoters, a splice acceptor and/or donor sequences, and termination and polyadenylation signals. Also required is the sequence for a ribosome binding site capable of permitting translation which is operably linked to the gene to be expressed. Recently, a muscle-specific promoter has been isolated which is positioned upstream of both the skeletal muscle structural gene and the essential proximal promoter element and is operably associated with each. (Mar and Ordahl, Proc. Natl. Acad. Sci. USA 85, 6404-6408 (1988)). Vectors comprise plasmids, viruses (e.g. adenovirus, cytomegalovirus), phage, and DNA fragments integratable

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into the host genome by recombination. The vector replicates and functions independently of the host genome, or may in some instances integrate into the genome itself.

5 The present invention is explained in greater detail in the following non-limiting Examples.

#### EXAMPLE 1

##### Preparation of Feeder Cells

10 Gelatinizing culture dishes are prepared as follows. First, 0.1% gelatin is added to water to prepare a gelatin solution, which is then autoclaved. 4 ml of the gelatin solution is added to each plate for 6 cm plates, or 2 ml/well of gelatin solution is added to each well for 12-well plates. The plates or wells are  
15 incubated at 4° C for 30 minutes, and the gelatin aspirated prior to use.

STO feeder cells (American Type Culture Collection No. CRL 1503) are prepared by culturing STO cells to 80% confluency in DMEM with 10% FBS. The cells  
20 are then treated with mitomycin C at 10 µg/ml for 2-3 hours, after which they are rinsed three times with PBS. After rinse, the cells are trypsinized with a .25% trypsin/.025% EDTA solution, the cells collected in DMEM with 10% FBS, and washed at 1,000 rpm for 5 min. After  
25 washing, cells are suspended in 5 ml of DMEM w.10% FBS and counted. The cells are then seeded onto gelatinized plates prepared as described above at a density of  $1 \times 10^5/\text{cm}^2$  and incubated overnight before use.

30 Primary Chicken Embryonic fibroblasts are prepared by harvesting fibroblasts from 10-day old chick embryos, subculturing the cells once, and then preparing the cells as feeder cells as listed for STO cells above.

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## EXAMPLE 2

Preparation of Conditioned Media

5 Buffalo Rat Liver (BRL) cell conditioned media is prepared by culturing BRL-3A cells (American Type Culture Collection No. CRL 1442) in DMEM w/10% FBS to confluency, then adding 13 ml of DMEM/10%FBS to each 75cm<sup>2</sup> flask. Media is collected from the flask every third day, with each flask being collected three to four times. Media is stored at -20° C. For use, the media is  
10 filtered, adjusted to pH 7.5 with HCl, diluted to 80% BRL-CM with DMEM supplemented with 15% FBS, and the diluted conditioned media then supplemented with 0.1 mM  $\beta$ -mercaptoethanol.

15 LMH (chicken liver cell) conditioned media is prepared by culturing LMH cells in the same manner as for BRL-3A cells above, and the conditioned media prepared in the same manner as BRL-conditioned media as given above.

## EXAMPLE 3

Isolation of Unincubated Chick Embryo Cells

20 To isolate stages IX-XIV embryo cells, the surface of a fertilized chicken egg is sterilized with 70% ethanol, the egg opened, and the yolk separated from the albumen. The yolk is then placed in a petri dish with the blastoderm in the uppermost position. A filter  
25 paper ring is placed over the blastoderm and the yolk membrane cut around the periphery of the ring. The filter paper ring with the embryo is then transferred to PBS with the ventral side uppermost, excess yolk removed, the embryo teased from the yolk membrane, the embryo  
30 transferred to cold PBS and rinsed with PBS. PBS is then removed, trypsin added, and the embryo incubated for 10 min. at 4° C. DMEM/10% FBS is added, the cells pelleted by centrifugation, the supernatant removed, and the cells resuspended in 80% BRL-CM. Embryo cells are then seeded  
35 onto the appropriate culture system.

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## EXAMPLE 4

Culturing of Avian Embryonic Stem Cells

Using the procedures given above several methods of culturing cell with an embryonic stem cell phenotype from unincubated chicken embryos were carried out.

First, 10 whole embryos at stage X were isolated, dissociated, seeded onto chicken embryonic fibroblast feeder layers, and cultured with 80% BRL-CM. A significant amount of differentiation occurred, mainly cells of a fibroblast-like phenotype. Only a few clusters of cells remained relatively undifferentiated and contained large amounts of lipid. These cells grew slowly, if at all, and were lost by the second passage.

Second, 10 whole embryos at stage X were isolated, dissociated, seeded onto STO feeder layers, and cultured with 80% BRL-CM. Upon culture, the cells attached to the feeder layer and grew as small flattened colonies. In the first 3 passages, the cells lost all lipid droplets and exhibited a phenotype and growth characteristics similar to that observed for murine and porcine embryonic stem cells. Specifically, the cells contained a large nucleus with a prominent nucleolus and relatively little cytoplasm (see Fig. 1 and Fig. 2). The cells grew in nests with a generally uniform phenotype. Each nest remained a single cell thick as it grew, a characteristic shared with porcine, but not murine, embryonic stem cells. Unlike either murine or porcine cells, the nests of chicken embryonic stem cells exhibited the tendency to invade the feeder layer, pushing the STO feeder cells to the side or growing underneath the feeder layer. It was possible to culture these cells for 23 passages. In general,  $10^6$  cells were seeded onto a STO feeder layer in a 6 cm dish and in 2-3 days  $2 \times 10^6$  to  $5 \times 10^7$  CES cells could be obtained.

On the fifth passage, a portion of the CES cells were transferred to BRL-CM media alone. Initially,

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the CES cells grew rapidly and formed large ES-like colonies. When passed onto new gelatinized plates in BRL-CM, the cells differentiated into fibroblast-like cells, accumulated lipid droplets and died with the next passage. Likewise, at the tenth passage a portion of the CES cells grown on STO cells and in BRL-CM were seeded onto STO feeder cells alone. These cells also became fibroblast-like, and could not be maintained on STO feeder cells alone. These observation suggest that the culture of chicken embryonic stem cells requires both a feeder layer and conditioned media.

## EXAMPLE 5

Initiating and Maintenance of  
Chicken Embryonic Stem (CES) Cells

Chicken embryonic stem cells are initiated by isolating stage IX-XIV unincubated chicken embryos, and the area pellucida used as the source of cells for culture. Cells are seeded onto mitotically inactivated STO feeder layers with 80% BRL-CM and DMEM supplemented with 15% FBS and .1 mM  $\beta$ -mercaptoethanol. After the initial seeding, the phenotype of the chicken cells is observed daily. Eventually, a portion of the cells begin to lose their lipid droplets and begin to invade the feeder layer while remaining a closely packed nest of cells generally one cell thick. During the first few passages, the entire culture is passed onto new STO feeder layers until several nests of stem cell-like cells appear.

Once an initial culture shows several nests of stem cells, the cultures are maintained by trypsinizing the culture and counting the number of chicken embryonic stem cells. About .3 to  $1 \times 10^6$  CES cells are seeded onto new STO feeder layers. The cultures are fed twice each day with BRL-CM and passed onto new feeder layers every 2-3 days, depending upon the density of the CES cells. Using this procedure, CES cells have been maintained for

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23 passages (approximately two months). Data are given in Table 1 below.

TABLE 1. Yield of CES cells with each passage. Chick embryo cells were seeded at  $3.5 \times 10^5$  cells/6 cm plate at day 0.  $1 \times 10^6$  cells were seeded with each passage.

Day of Culture	Passage Number	Total CES X $10^6$
23	9	3.0
25	10	3.5
27	11	4.0
29	12	2.0
31	13	1.5
34	14	3.0
37	15	1.3
38	16	2.0
42	17	2.5
44	18	3.0
49	19	1.0
51	20	1.6
53	21	.7

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.



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**THAT WHICH IS CLAIMED IS:**

1. A method of producing undifferentiated avian cells expressing an embryonic stem cell phenotype, comprising:

5           collecting avian cells from an avian blastoderm prior to formation of the primitive streak;

          depositing said avian cells in contact with a mouse fibroblast feeder cell layer;

          growing said avian cells on said mouse  
10   fibroblast feeder cell layer in the presence of a media containing leukemia inhibitory factor in a differentiation-inhibiting amount for a time sufficient to produce a sustained avian cell culture;

          said sustained avian cell culture consisting  
15   essentially of undifferentiated avian cells having a large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype").

2. A method according to claim 1, wherein said  
20   avian cells are collected from the area pellucida of a stage IX to stage XIV blastoderm.

3. A method according to claim 1, wherein said  
undifferentiated avian cells are capable of maintaining  
said stem cell phenotype when grown on said mouse  
fibroblast feeder layer in the presence of said media for  
25   at least three days.

4. A method according to claim 1, wherein said  
avian blastoderm is a blastoderm selected from the group  
consisting of chicken, turkey, duck, geese, quail and  
pheasant blastoderms.

30           5. A method according to claim 1, wherein said  
avian blastoderm is a chicken blastoderm.

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6. A method according to claim 1, wherein said media is a conditioned media.

7. A method according to claim 1, wherein said mouse fibroblast feeder cell layer is a mouse STO fibroblast feeder cell layer.

8. An avian cell culture produced by the process of claim 1.

9. A method of producing undifferentiated chicken cells expressing an embryonic stem cell phenotype, comprising:

collecting avian cells from the area pellucida of a stage IX to stage XIV chicken blastoderm;

depositing said avian cells in contact with a mouse STO fibroblast feeder cell layer;

growing said avian cells on said mouse fibroblast feeder cell layer in the presence of a conditioned media containing leukemia inhibitory factor in a differentiation-inhibiting amount for a time sufficient to produce a sustained avian cell culture;

said sustained avian cell culture consisting essentially of undifferentiated avian cells having a large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype"), and wherein said undifferentiated avian cells are capable of maintaining said stem cell phenotype when grown on said mouse fibroblast feeder layer in the presence of said media for at least three days.

10. A chicken cell culture produced by the process of claim 9.

11. A sustained avian cell culture consisting essentially of undifferentiated avian cells having a

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large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype").

12. A sustained avian cell culture according to claim 11, wherein said cells are produced from ancestor cells isolated from an avian blastoderm prior to formation of the primitive streak.

13. A sustained avian cell culture according to claim 11, wherein said cells are produced from ancestor cells isolated from the area pellucida of a stage IX to stage XIV avian blastoderm.

14. A sustained avian cell culture according to claim 11, wherein said undifferentiated avian cells are capable of maintaining said stem cell phenotype when grown on a mouse fibroblast feeder layer in the presence of a media containing leukemia inhibitory factor in a differentiation-inhibiting amount for at least three days.

15. A sustained avian cell culture according to claim 11, wherein said avian cells are chicken cells.

16. A veterinary pharmaceutical formulation comprising sustained avian cell in a pharmaceutically acceptable carrier, said sustained avian cells comprising undifferentiated avian cells having a large nucleus, a prominent nucleolus, and little cytoplasm (an "embryonic stem cell phenotype").

17. A veterinary pharmaceutical formulation according to claim 16, wherein said cells are produced from ancestor cells isolated from an avian blastoderm prior to formation of the primitive streak.

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18. A veterinary pharmaceutical formulation according to claim 16, wherein said cells are produced from ancestor cells isolated from the area pellucida of a stage IX to stage XIV avian blastoderm.

5 19. A veterinary pharmaceutical formulation accordin to claim 16, wherein said undifferentiated avian cells are capable of maintaining said stem cell phenotype when grown on a mouse fibroblast feeder layer in the presence of a media containing leukemia inhibitory factor  
10 in a differentiation-inhibiting amount for at least three days.

20. A veterinary pharmaceutical formulation according to claim 16, wherein said avian cells are chicken cells.

I/I



**FIG. 1**



**FIG 2**

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04581

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C12N 5/00; A61K 37/00

US CL : 435/240.2, 240.21, 240.23, 240.3, 240.31; 424/93U

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 240.23, 240.3, 240.31; 424/93U

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Roux's Arch. Dev. Biol., Volume 198, issued 1989, Handyside et al, "Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line", pages 48-56, see entire document.	1-20
Y	Developmental Biology, Volume 141, issued 1990, Pease et al, "Isolation of Embryonic Stem (ES) Cells in Media Supplemented with Recombinant Leukemia Inhibitory Factor (LIF)", pages 344-352, see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

19 AUG 1993

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## INTERNATIONAL SEARCH REPORT

International application No.  
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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell Tissue Research, Volume 220, issued 1981, Sanders et al, "Morphological differentiation of an embryonic epithelium in culture", pages 539-548, see entire document.	1-20
Y	Differentiation, Volume 21, issued 1982, Mitrani et al, "Cells from Early Chick Embryos in Culture", pages 56-61, see entire document.	1-20
A	Journal of Cellular Biochemistry, Supplement 13B, issued 1989, "The Production of Chimeric Chicks by Embryonic Cell Transfer", page 172, see abstract F114.	1-20
A	WO, A, 90/03432 (Evans et al) 05 April 1990, see entire document.	1-20